

Serial No.: 09/800,770

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IN THE SPECIFICATION

Please replace the section titled "BRIEF DESCRIPTION OF THE DRAWINGS", with the following rewritten section:

– Figure 1A depicts head to tail protein cyclization by reconfigured/engineered intein.

Figure 1B depicts the mechanism of cyclization by reconfigured/engineered intein.

Figure 2A depicts intein catalyzed ligation by the Mxe GyrA intein. In it's normal configuration, intein catalyzed ligation joins the extein residues located at the junction points with each of the two intein motifs.

Figure 2B depicts the outcome of a motif reorganization resulting in the production of a cyclic peptide. Motif reorganiziation involves providing intein B with its own translational start codon and placing intein B amino-terminal to intein A.

Figure 3A (SEQ ID NO:1) depicts the amino acid sequence of intein Ssp DnaB from *Synechocystis sp.* strain PCC6803.

Figure 3B (SEQ ID NO:2) depicts the amino acid sequence of intein Mxe GyrA from *Mycobacterium xenopi*.

Figure 3C (SEQ ID NO:3) depicts the amino acid sequence of intein Ceu ClpP from *Chlamydomonas eugametos*.

Figure 3D (SEQ ID NO:4) depicts the amino acid sequence of intein CIV RIR1 from Chilo iridescent virus.

Figure 3E (SEQ ID NO:5) depicts the amino acid sequence of intein Ctr VMA from *Candida tropicalis*.

Figure 3F (SEQ ID NO:6) depicts the amino acid sequence of intein Gth DnaB from *Guillardia theta*.

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Figure 3G (SEQ ID NO:7) depicts the amino acid sequence of intein Ppu DnaB from *Porphyra purpurea*.

Figure 3H (SEQ ID NO:8) depicts the amino acid sequence of intein Sce VMA from *Saccharomyces cerevisiae*.

Figure 3I (SEQ ID NO:9) depicts the amino acid sequence of intein Mf1 RecA from *Mycobacterium flavescens*.

Figure 3J (SEQ ID NO:10) depicts the amino acid sequence of intein Ssp DnaE from *Synechocystis sp.* strain PCC6803.

Figure 3K (SEQ ID NO:11) depicts the amino acid sequence of intein Mle DnaB from *Mycobacterium leprae*.

Figure 3L (SEQ ID NO:12) depicts the amino acid sequence of intein Mja KlbA from *Methanococcus jannaschii*.

Figure 3M (SEQ ID NO:13) depicts the amino acid sequence of intein Pfu KlbA from *Pyrococcus furiosus*.

Figure 3N (SEQ ID NO:14) depicts the amino acid sequence of intein Mth RIR1 from *Methanobacterium thermoautotrophicum* (delta H strain).

Figure 3O (SEQ ID NO:15) depicts the amino acid sequence of intein Pfu RIR1-1 from *Pyrococcus furiosus*.

Figure 3P (SEQ ID NO:16) depicts the amino acid sequence of intein Psp-GBD Pol from *Pyrococcus sp.* GB-D.

Figure 3Q (SEQ ID NO:17) depicts the amino acid sequence of intein Thy Pol-2 from *Thermococcus hydrothermalis*.

Figure 3R (SEQ ID NO:18) depicts the amino acid sequence of intein Pfu IF2 from *Pyrococcus furiosus*.

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Figure 3S (SEQ ID NO:19) depicts the amino acid sequence of intein Pho Lon from *Pyrococcus horikoshii* OT3.

Figure 3T (SEQ ID NO:20) depicts the amino acid sequence of intein Mja r-Gyr from *Methanococcus jannaschii*.

Figure 3U (SEQ ID NO:21) depicts the amino acid sequence of intein Pho RFC from *Pyrococcus horikoshii* OT3.

Figure 3V (SEQ ID NO:22) depicts the amino acid sequence of intein Pab RFC-2 from *Pyrococcus abyssi*.

Figure 3W (SEQ ID NO:23) depicts the amino acid sequence of intein Mja RtcB (Mja Hyp-2) from *Methanococcus jannaschii*.

Figure 3X (SEQ ID NO:24) depicts the amino acid sequence of intein Pho VMA from *Pyrococcus horikoshii* OT3.

Figure 4A (SEQ ID NOS:25 & 26) depicts the amino acid sequence of a modified wild-type Ssp DnaB Intein. The DNA sequence is provided in Figures 4B-1 and 4B-2 (SEQ ID NOS:27 & 28).

Figures 5A and B (SEQ ID NOS:29 & 30) depict the nucleotide and amino acid sequence of the intein Ssp DnaB J3 template used to generate intein mutants L7-J3, E6-J3, E9-J3, C11-J3 and B8-J3, with improved splicing efficiency. The J3 template carries a mutation which results in a amino acid change D to N at position 320. Thus, all mutants based on the J3 template are double mutants.

Figures 5C and D (SEQ ID NOS:31 & 32) depict the nucleotide and amino acid sequence of intein mutant L7-J3. L7 has two mutations which result in amino acid changes: 1) D to N at position 320 and 2) R to K at position 389.

Figures 5E and F (SEQ ID NOS:33 & 34) depict the nucleotide and amino acid sequence of intein mutant E6-J3. E6 has two mutations which result in amino acid changes: 1) D to N at position 320 and 2) I to V at position 34.

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Figures 5G and H (SEQ ID NOS:35 & 36) depict the nucleotide and amino acid sequence of intein mutant E9-J3. E9 has two mutations which result in amino acid changes: 1) D to N at position 320 and 2) T to A at position 36.

Figures 5I and J (SEQ ID NOS:37 & 38) depict the nucleotide and amino acid sequence of intein mutant C11-J3. C11 has two mutations which result in amino acid changes: 1) D to N at position 320 and 2) S to P at position 23.

Figures 5K and L (SEQ ID NOS:39 & 40) depict the nucleotide and amino acid sequence of intein mutant B8-J3. B8 has two mutations which result in amino acid changes: 1) D to N at position 320 and 2) K to R at position 369.

Figures 5M and N (SEQ ID NOS:41 & 42) depict the nucleotide and amino acid sequence of intein mutant L7-wt, which was generated from an Ssp DnaB wild-type (wt) template. Mutants generated from the wt template carry a single mutation which effects splicing efficiency. L7-wt carries a single mutation which results in the amino acid change R to K at position 389.

Figures 5O and P (SEQ ID NOS:43 & 44) depict the nucleotide and amino acid sequence of intein mutant C11-wt. C11-wt has a single mutation which result in the amino acid change S to P at position 23.

Figures 5Q and R (SEQ ID NOS:45 & 46) depict the nucleotide and amino acid sequence of intein mutant E6-wt. E6-wt has a single mutation which result in the amino acid change I to V at position 34.

Figures 6A-6D (SEQ ID NOS:47 & 48) depict the DNA sequence for a N-terminally fused GFP version of the Ssp DnaB intein.

Figure 7 depicts reporter proteins which can be used for the selection and/or detection of intein-based libraries.

Figure 8 depicts localization sequences which can be used to target cyclic peptide libraries.

Figure 9 depicts a random mutagenesis approach used in the optimization of intein cyclization function.

Figure 10 depicts a biotinylation approach for use in a yeast two hybrid system.

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Figure 11 depicts a single chain antibody approach for use in a yeast two hybrid system.

Figure 12 depicts the fluorescent reporter system used to quantify intein cyclization. Figure 12 A depicts GFP split at the loop 3 junction and reversal of the translation order of the N- and C-terminal fragments. The termini are fused using a glycine-serine linker. The GFP is positioned within the Ssp DnaB intein cyclizationscaffold. Cyclized product reconstitutes both structure and fluorescence of GFP. In addition, splicing one-half of the myc epitope onto either side of the loop 3 junction allows for reconstruction of the myc epitope upon cyclization.

Figure 12B (SEQ ID NO:49) provides the amino acid sequence of DNAB intein cyclization scaffold with GFP.

Figure 12C (SEQ ID NOS:50-53) depicts the mechanism of intein catalyzed cyclization of inverted loop 3 of GFP.

Figure 12D shows the results from a FACS analysis of the cyclization efficiency of wild-type Ssp DnaB intein in mammalian cells.

Figure 12E shows the results from a Western analysis of a Ssp DnaB catalyzed cyclization in mammalian cells.

Figure 12F shows the results from a native gel and the contribution to GFP fluorescence. The majority of the fluorescence arises from the formation of cyclized GFP product, bands C and D.

Figure 13 illustrates a functional screen for isolating randomly-generated mutants with altered cyclization activity. Figure 13A depicts a functional screen for intein mutants with altered cyclization activity. Figure 13B depicts mutations modeled on the Mxe GyrA intein structure. Figure 13C (SEQ ID NOS:54 & 55) depicts the sequence alignment of Mxe GyrA (SEQ ID NO:55) and Ssp DnaB inteins (SEQ ID NO:54). Mutants are identified in shaded color. Figure 13D shows the results from a western analysis of isolated mutants. DnaB mutants E9-J3, E6-J3, C11-J3, L7-J3, and B8-J3 have cyclization efficiencies that are greater than the J3 starting intein template.

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Figure 14 depicts intein-mediated excision/ligation in mammalian cells. Figure 14 A depicts constructs in which Ssp DnaB intein is inserted into loop 3 of GFP (i.e., GAB) or GFP with a C-terminal myc epitope. Figure 14B depicts constructs similar to those shown in 14A, except that the myc epitope (SEQ ID NO:56) half-sites are positioned onto the extreme ends of each splice junction. Figure 14C depicts Western blot analysis of lysates from transfected Phoenix cells. Lanes 3 and 4 demonstrate efficient splicing with only slight amounts of unspliced product detected.

Figures 15A-D depict a method for detecting cyclic peptides in mammalian cells. Figure 15A (SEQ ID NOS:57 & 58) depicts an overview of the method in which cyclic peptides are detected in mammalian cells expressing a GFP fused intein scaffold with cyclic peptide inserts. Figures 15 B and C depict the MS analysis of mammalian cell lysates expressing the cyclic peptide products from RGD7 (15B) and RGD9 (15C). Figure 15D depicts an example of LC/MS fragmentation fingerprinting of the cyclic peptide product of an intein construct.

Figure 16 depicts the low energy conformers associated with cyclic peptide SRGDGWS (SEQ ID NO:57).

Figure 17 depicts the low energy conformers associated with cyclic peptide SRGPGWS (SEQ ID NO:59).—

In the Section titled “DETAILED DESCRIPTION OF THE INVENTION,” please replace the paragraph beginning at page 10, line 16, with the following rewritten paragraph:

— Preferred fusion polypeptides of the invention increase the efficiency of the cyclization reaction by selecting or designing intein motifs with altered cyclization activity when expressed *in vivo*. In a preferred embodiment, the fusion polypeptides of the invention employ the DNA sequence encoding the *Synechocystis ssp.* strain PCC6803 DnaB intein. A particularly preferred fusion polypeptide structure is illustrated in Figure 4A and 4B (SEQ ID NOS:25-28).—

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Please replace the paragraph beginning at page 10, line 22, with the following rewritten paragraph:

– In a preferred embodiment, fusion polypeptides are designed using mutant intein sequences with altered cyclization activity as described below. Preferred mutant intein sequences include, but are not limited, to those shown in Figure 5 (SEQ ID NOS:29-46).—

Please replace the paragraph beginning at page 17, line 36, with the following rewritten paragraph:

– In a preferred embodiment, the targeting sequence is a nuclear localization signal (NLS). NLSes are generally short, positively charged (basic) domains that serve to direct the entire protein in which they occur to the cell's nucleus. Numerous NLS amino acid sequences have been reported including single basic NLSes such as that of the SV40 (monkey virus) large T Antigen (Pro Lys Lys Lys Arg Lys Val (SEQ ID NO:60)), Kalderon (1984), et al., Cell, 39:499-509; the human retinoic acid receptor- β nuclear localization signal (ARRRRP (SEQ ID NO:61)); NF κ B p50 (EEVQRKRQKL (SEQ ID NO:62); Ghosh et al., Cell 62:1019 (1990); NF κ B p65 (EEKRKRTYE (SEQ ID NO:63); Nolan et al., Cell 64:961 (1991); and others (see for example Boulikas, J. Cell. Biochem. 55(1):32-58 (1994), hereby incorporated by reference) and double basic NLSes exemplified by that of the Xenopus (African clawed toad) protein, nucleoplasmin (Ala Val Lys Arg Pro Ala Ala Thr Lys Lys Ala Gly Gln Ala Lys Lys Lys Lys Leu Asp (SEQ ID NO:64)), Dingwall, et al., Cell, 30:449-458, 1982 and Dingwall, et al., J. Cell Biol., 107:641-849; 1988). Numerous localization studies have demonstrated that NLSes incorporated in synthetic peptides or grafted onto reporter proteins not normally targeted to the cell nucleus cause these peptides and reporter proteins to be concentrated in the nucleus. See, for example, Dingwall, and Laskey, Ann. Rev. Cell Biol., 2:367-390, 1986; Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799, 1987; Galileo, et al., Proc. Natl. Acad. Sci. USA, 87:458-462, 1990.—

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Please replace the paragraph beginning at page 19, line 12, with the following rewritten paragraph:

– Useful sequences include sequences from: 1) class I integral membrane proteins such as IL-2 receptor β -chain (residues 1-26 are the signal sequence, 241-265 are the transmembrane residues; see Hatakeyama et al., Science 244:551 (1989) and von Heijne et al, Eur. J. Biochem. 174:671 (1988)) and insulin receptor β -chain (residues 1-27 are the signal, 957-959 are the transmembrane domain and 960-1382 are the cytoplasmic domain; see Hatakeyama, supra, and Ebina et al., Cell 40:747 (1985)); 2) class II integral membrane proteins such as neutral endopeptidase (residues 29-51 are the transmembrane domain, 2-28 are the cytoplasmic domain; see Malfroy et al., Biochem. Biophys. Res. Commun. 144:59 (1987)); 3) type III proteins such as human cytochrome P450 NF25 (Hatakeyama, supra); and 4) type IV proteins such as human P-glycoprotein (Hatakeyama, supra). Particularly preferred are CD8 and ICAM-2. For example, the signal sequences from CD8 and ICAM-2 lie at the extreme 5' end of the transcript. These consist of the amino acids 1-32 in the case of CD8 (MASPLTRFLSLNLLLLGESILGSGEAKPQAP (SEQ ID NO:65); Nakauchi et al., PNAS USA 82:5126 (1985) and 1-21 in the case of ICAM-2 (MSSFGYRTLTVLFTLICCPG (SEQ ID NO:66); Staunton et al., Nature (London) 339:61 (1989)). These leader sequences deliver the construct to the membrane while the hydrophobic transmembrane domains, placed 3' of the random peptide region, serve to anchor the construct in the membrane. These transmembrane domains are encompassed by amino acids 145-195 from CD8 (PQRPEDCRPRGSVKGTGLDFACDIYWAPLAGICVALLLSLIITLICYHSR (SEQ ID NO:67); Nakauchi, supra) and 224-256 from ICAM-2 (MVIIVTVSVLLSLFVTSVLLCFIFGQHRLRQQR (SEQ ID NO:68); Staunton, supra).–

Please replace the paragraph beginning at page 19, line 31, with the following rewritten paragraph:

– Alternatively, membrane anchoring sequences include the GPI anchor, which results in a covalent bond between the molecule and the lipid bilayer via a glycosyl-phosphatidylinositol bond for example in DAF (PNKGS~~GT~~**TS**GTTRLLSGHTCFTLTGLLGTLVTMGLLT (SEQ ID NO:69), with the bolded serine the site of the anchor; see Homans et al., Nature 333(6170):269-72 (1988), and Moran et al., J. Biol. Chem. 266:1250 (1991)). In order to do this, the GPI sequence from Thy-1 can be cassetted 3' of the variable region in place of a transmembrane sequence.–

Please replace the paragraph beginning at page 20, line 1, with the following rewritten paragraph:

– Similarly, myristylation sequences can serve as membrane anchoring sequences. It is known that the myristylation of c-src recruits it to the plasma membrane. This is a simple and effective method of membrane localization, given that the first 14 amino acids of the protein are solely responsible for this function: MGSSKSKPKDPSQR (SEQ ID NO:70) (see Cross et al., Mol. Cell. Biol. 4(9):1834 (1984); Spencer et al., Science 262:1019-1024 (1993), both of which are hereby incorporated by reference). This motif has already been shown to be effective in the localization of reporter genes and can be used to anchor the zeta chain of the TCR. This motif is placed 5' of the variable region in order to localize the construct to the plasma membrane. Other modifications such as palmitoylation can be used to anchor constructs in the plasma membrane; for example, palmitoylation sequences from the G protein-coupled receptor kinase GRK6 sequence (LLQRLFSRQ**DCC**GNCS**DSE**EELPT**RL** (SEQ ID NO:71), with the bold cysteines being palmitoylated; Stoffel et al., J. Biol. Chem 269:27791 (1994)); from rhodopsin (KQFRNCMLTSL**CC**GKNPLGD (SEQ ID NO:72); Barnstable et al., J. Mol. Neurosci. 5(3):207 (1994)); and the p21 H-ras 1 protein (LNPPDESGPGCM**SCK**CVLS (SEQ ID NO:73); Capon et al., Nature 302:33 (1983)).–

Please replace the paragraph beginning at page 20, line 15, with the following rewritten paragraph:

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– In a preferred embodiment, the targeting sequence is a lysosomal targeting sequence, including, for example, a lysosomal degradation sequence such as Lamp-2 (KFERQ (SEQ ID NO:74); Dice, Ann. N.Y. Acad. Sci. 674:58 (1992); or lysosomal membrane sequences from Lamp-1 (*MLIPIAGFFALAGLVLI**VLIAYLIGRK**RSHAGYQTI* (SEQ ID NO:75), Uthayakumar et al., Cell. Mol. Biol. Res. 41:405 (1995)) or Lamp-2 (*LVPIAVGAALAGVL**LVLLAYFI**GLKHHHAGYE**QF* (SEQ ID NO:76), Konecki et al., Biochem. Biophys. Res. Comm. 205:1-5 (1994), both of which show the transmembrane domains in italics and the cytoplasmic targeting signal underlined).–

Please replace the paragraph beginning at page 20, line 23, with the following rewritten paragraph:

– Alternatively, the targeting sequence may be a mitochondrial localization sequence, including mitochondrial matrix sequences (e.g. yeast alcohol dehydrogenase III; MLRTSSLFTRRVQPSLFSRNILRLQST (SEQ ID NO:77); Schatz, Eur. J. Biochem. 165:1-6 (1987)); mitochondrial inner membrane sequences (yeast cytochrome c oxidase subunit IV; MLSLRQSIRFFKPATRTLCSRYLL (SEQ ID NO:78); Schatz, supra); mitochondrial intermembrane space sequences (yeast cytochrome c1; MFSMLSKRWAQRTLKSFYSTATGAASKSGKLTQKLVTAGVAAAGITASTLLYADSLTAEAMTA (SEQ ID NO:79); Schatz, supra) or mitochondrial outer membrane sequences (yeast 70 kD outer membrane protein; MKSFITRNKTAILATVAATGTAIGAYYYNQLQQQQQRGKK (SEQ ID NO:80); Schatz, supra).–

Please replace the paragraph beginning at page 20, line 32, with the following rewritten paragraph:

– The target sequences may also be endoplasmic reticulum sequences, including the sequences from calreticulin (KDEL (SEQ ID NO:81); Pelham, Royal Society London Transactions B; 1-10 (1992)) or

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adenovirus E3/19K protein (LYLSRRSFIDEKKMP (SEQ ID NO:82); Jackson et al., EMBO J. 9:3153 (1990)).—

Please replace the paragraph beginning at page 20, line 36, with the following rewritten paragraph:

– Furthermore, targeting sequences also include peroxisome sequences (for example, the peroxisome matrix sequence from Luciferase; SKL; Keller et al., PNAS USA 4:3264 (1987)); farnesylation sequences (for example, P21 H-ras 1; LNPPDESGPGCMSCKCVLS (SEQ ID NO:83), with the bold cysteine farnesylated; Capon, supra); geranylgeranylation sequences (for example, protein rab-5A; LTEPTQPTRNQCCSN (SEQ ID NO:84), with the bold cysteines geranylgeranylated; Farnsworth, PNAS USA 91:11963 (1994)); or destruction sequences (cyclin B1; RTALGDIGN (SEQ ID NO:85); Klotzbucher et al., EMBO J. 1:3053 (1996)).—

Please replace the paragraph beginning at page 21, line 25, with the following rewritten paragraph:

– Suitable secretory sequences are known, including signals from IL-2 (MYRMQLLSICIALSLALVTNS (SEQ ID NO:86); Villinger et al., J. Immunol. 155:3946 (1995)), growth hormone (MATGSRTSLLAFGLLCLPWLQEGSAFPT (SEQ ID NO:87); Roskam et al., Nucleic Acids Res. 7:305 (1979)); preproinsulin (MALWMRLLPLLALLALWGPDPAAAFVN (SEQ ID NO:88); Bell et al., Nature 284:26 (1980)); and influenza HA protein (MKAKLLVLLYAFVAGDQI (SEQ ID NO:89); Sekikawa et al., PNAS 80:3563), with cleavage between the non-underlined-underlined junction. A particularly preferred secretory signal sequence is the signal leader sequence from the secreted cytokine IL-4, which comprises the first 24 amino acids of IL-4 as follows: MGLTSQLLPPLFFLLACAGNFVHG (SEQ ID NO:90).—

Please replace the paragraph beginning at page 59, line 26, with the following rewritten paragraph:

– To test the effects of a fixed proline in a cyclic 7mer, the conformation space of the 7mer cyclic peptide SRGDGWS (SEQ ID NO:57), containing two flexible glycines was compared with that of cyclic SRPGWS (SEQ ID NO:59) using quenched molecular dynamics calculations (O'Connor, et al., (1992) J. Med. Chem., 35:2870-81); Mackay, et al., (1989) "The role of energy minimization in simulation strategies of biomolecular systems", In *Prediction of Protein Structure and the Principles of Protein Conformation*, Fasman, G., ed., New York, Plenum Press, pp. 317-358).–

Please replace the paragraph beginning at page 60, line 1, with the following rewritten paragraph:

– An example of the cluster graph of the lowest energy conformers for each peptide is shown in Figures 16 and 17. The root mean square deviation (RMSD, Å) is coded by color, with very similar conformers ($\text{RMSD} \leq \text{Å}$) in yellow, still highly similar conformers (RMSD between 1-2 Å) in white, similar conformers (RMSD between 2-3 Å) in blue, less similar conformers (RMSD between 3-4 Å) in red, and dissimilar conformers in black (not shown).–

Please replace the paragraph beginning at page 60, line 7, with the following rewritten paragraph:

– For the cyclic peptide SRGDGWS (SEQ ID NO:57), shown in Figure 16 (srgdgwsLowest5A.ps), there were 62 low energy conformers. There was one family of very similar conformers (yellow square at bottom left) and two families of quite similar conformers in yellow/white, one roughly in the middle of the graph, and one (with only moderately similar conformers) near the top right corner. These comprised approximately 20 of the 62 conformers. The rest of the low energy conformers were not very similar to each other, and much of the graph is red or black. Backbone overlaid conformers from most similar family, No. 1, are shown at the lower left. In the lower middle, is family No. 2. these conformers, when